L-URIDINE: SYNTHESIS AND BEHAVIOR AS ENZYME SUBSTRATE*

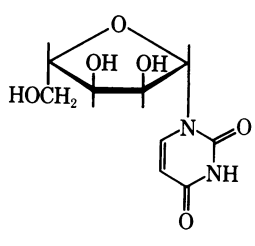
By Anna Fang Wu and Erwin Chargaff

CELL CHEMISTRY LABORATORY, DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, NEW YORK

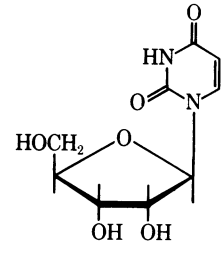
Communicated May 16, 1969

Abstract.—L-Uridine, the enantiomer of the normal RNA constituent D-uridine, was synthesized from L-ribose through coupling with bis(trimethylsilyl)-uracil. The synthetic product had the expected chemical and physical characteristics. When used as the acceptor for phosphate transfer by the nucleoside phosphotransferase of carrot, L-uridine is converted to 5′-L-uridylic acid. The Michaelis constants K_m are 28×10^{-3} M for L-uridine, 5×10^{-3} M for D-uridine. The nucleoside phosphotransferase of human prostate, which phosphorylates D-uridine in the 5′, 3′, or 2′ positions, fails to transfer phosphate to the 2′ position of L-uridine, but does produce 5′- and 3′-L-uridylic acids.

The ribonucleosides, which are linked by phosphodiester bridges to form RNA, are furanoside derivatives of D-ribose. In connection with various interests of this laboratory, which will be mentioned below, we were anxious to study some of the biological properties of the enantiomeric "unnatural" nucleoside and nucleotide derivatives of L-ribose. We describe in this communication the complete synthesis of L-uridine (I) and compare it with the normal RNA constituent D-uridine (II) with regard to its function as acceptor nucleoside for two different nucleoside phosphotransferases, namely, those of carrot and human prostate. This paper, therefore, touches also on the enzymic synthesis of two enantiomers of the uridylic acid isomers normally obtainable from RNA, viz., 3′- and 5′-L-uridylic acids.



(I)



(II)

Materials.—Chemicals: The commercial preparations of sodium phenylphosphate (Eastman Kodak), L-arabinose (Pfanstiehl), uracil and 5′-D-uridylic acid (Schwarz Bio-Research), 2′- and 3′-D-uridylic acids (P-L Biochemicals), and D-uridine (Calbiochem) were, when necessary, purified in the laboratory. All other organic reagents were supplied by Fisher Scientific Co.

Enzymes: Nucleoside phosphotransferase from carrot was prepared by a previously published procedure.[1] Nucleoside phosphotransferase from human prostate[2] was kindly given to us by Dr. E. F. Brunngraber.

Procedures.—The elementary analyses were performed by Galbraith Laboratories, Knoxville, Tennessee. Optical rotation was measured with a Schmidt and Haensch polarimeter. Melting points were determined in the Thomas-Hoover apparatus with suitable temperature corrections. Descending paper chromatography made use of Whatman No. 1 filter paper and of a solvent system consisting of *n*-propanol, concentrated ammonia, and water (11:7:2, v/v/v). The Picker Nuclear LCS-1000 nucleic acid analyzer proved very useful for the determination of the relative proportions of the isomeric 2′-, 3′-, and 5′-uridylic acids of both the D and L series.

Synthesis.—L-Ribose: This sugar was prepared from L-arabinose via L-arabinal. A recent method[3] was followed, except that for the saponification of diacetyl arabinal 0.5 *M* barium methoxide, and not 0.5 *N* solution as specified in this reference, was used. From 100 gm of L-arabinose, 9 gm of L-ribose were obtained as a thick syrup.

1-O-acetyl-2,3,5-tri-O-benzoyl-L-ribose: A solution of 2.9 gm of L-ribose in 63 ml of anhydrous methanol was mixed with 1.2 ml of a 21% solution of anhydrous HCl in anhydrous methanol and the mixture was stirred at room temperature for $3^1/_2$ hr. Six milliliters of dry pyridine were added and the solvent was evaporated *in vacuo*, and this process was repeated once more. To a solution of the residual oil in 34 ml of dry pyridine and 16 ml of dry chloroform, kept in an ice bath, 11.3 ml of benzoyl chloride were added by drops, the mixture was stirred in the cold overnight, poured on ice with continued stirring, and extracted three times with chloroform. The chloroform extract was washed with water, dried (Na_2SO_4), and twice evaporated *in vacuo*, the second time after the addition of toluene. The residue was dissolved in 4.6 ml of glacial acetic acid and 10.8 ml of acetic anhydride. The chilled solution was, after the dropwise addition of 1.6 ml of concentrated H_2SO_4, kept in the cold for 18 hr. The mixture was then poured on ice and extracted several times with chloroform. The extract was washed with several changes of water, with 5% $NaHCO_3$ and again with water, and dried ($MgSO_4$). The residue remaining on evaporation was recrystallized twice from hot isopropanol. The yield of acetyltribenzoylribose was 3.5 gm; melting point (corrected) 124.5–127.5°; $[\alpha]_D^{25} = -41.6°$ (c = 1.32, $CHCl_3$). (The literature reports for this compound:[4] melting point, 130–131°; $[\alpha]_D^{25} = -43.6°$. For the corresponding D-ribose derivative these figures are:[5] melting point, 131–132°; $[\alpha]_D^{25} = +42.2°$.) *Analysis:* Calculated for $C_{28}H_{24}O_9$ (504.50); C, 66.66; H, 4.79. Found: C, 66.38; H, 4.68.

1-(2,3,5-Tri-O-benzoyl-L-ribofuranosyl) uracil: A sample of 2.3 gm of the above described 1-O-acetyl-2,3,5-tri-O-benzoyl-L-ribose was converted to the 1-chloro derivative following the procedure customary for the D isomer.[6] The 1-chloro compound was mixed with 1.7 gm of bis(trimethylsilyl)uracil[7] and was heated at 190° for 45 min. To the cooled mixture 34 ml of 70% ethanol were added, the solvent was removed *in vacuo*, and the residue was treated with a small amount of hot benzene; insoluble uracil was removed by filtration, and the benzene solution stored in the cold. The resulting precipitate was recrystallized several times from benzene yielding 0.78 gm of the tribenzoyl-L-uridine: melting point (corrected) 142–143°; $[\alpha]_D^{26} = +44°$ (c = 1.9, $CHCl_3$). (The literature for the D-isomer[8] gives: melting point 144–145°; $[\alpha]_D^{29}$ −48° ($CHCl_3$).) *Analysis:* Calculated for $C_{30}H_{24}O_9N_2$ (556.52): C, 64.75; H, 4.35; N, 5.03. Found: C, 64.58; H, 4.46; N (Dumas), 5.03.

L-Uridine (L-ribofuranosyl uracil): L-uridine was obtained in very good yield from the tribenzoyl derivative described above by treatment with 2 *N* sodium methoxide in the arrangement previously described for D-uridine.[8] L-Uridine was recrystallized from 97% ethanol; the white crystals had melting point (corrected) 162.5–164.5° and $[\alpha]_D^{25} = -7.5°$ (c = 2.0, H_2O). (A sample of authentic recrystallized D-uridine showed melting point (corrected) 163.5–165° and $[\alpha]_D^{25} = +7.4°$ (c = 2.0, H_2O).) *Analysis:* Calculated for $C_9H_{12}O_6N_2$ (244.21): C, 44.26; H, 4.95; N, 11.47. Found: C, 44.10; H, 4.86; N (Dumas), 11.34.

A comparison of the spectral and other physical characteristics determined by us for L-uridine and D-uridine with those given in the literature for D-uridine will be found in Table 1.

TABLE 1. *Physical characteristics of* L-*uridine and* D-*uridine.*

		L-Uridine	D-Uridine		
			Found	Literature	Reference
Melting point		162.5–164.5° (corrected)	163.5–165° (corrected)	165° (uncorrected)	9
Optical rotation (H_2O)		−7.5° (t = 25°)	+7.4° (t = 25°)	+4.0° (t = 20°)	9
Spectral properties	λ_{max}	262	262	262	10
	ϵ_{max}	10,300	10,300	10,100	10
	ϵ_{260}	10,100	9,900	9,950	10
	A_{250}/A_{260}	0.75	0.75	0.74	11
	A_{280}/A_{260}	0.33	0.34	0.35	11
	A_{290}/A_{260}	0.03	0.03	0.03	11

L-Uridine as phosphate acceptor for nucleoside phosphotransferases: Carrot enzyme: The experimental arrangements were patterned after a previous paper.[1] Each assay tube contained 20 μmoles of sodium phenylphosphate as phosphate donor, L- or D-uridine as the acceptor, and purified enzyme[1] in a total volume of 0.2 ml of 0.1 *M* sodium acetate buffer of pH 5.0. Prior to the determination of the Michaelis constants, preliminary experiments served to define the quantity of enzyme required in order to remain within the initial linear range of product formation. The mixtures were incubated at 37° for 7 min. The nucleotides produced were determined by paper chromatography and ultraviolet spectrophotometry.

Prostate enzyme: Each assay tube contained 20 μmoles of sodium phenylphosphate, 3 μmoles of D- or L-uridine, and 0.1 ml of enzyme solution (corresponding to 340 μg of enzyme protein) in a total volume of 0.2 ml of acetate buffer of pH 5.0. The mixtures were incubated at 37° for various periods and the nucleotides formed were first isolated by paper chromatography. Since the various isomeric uridylic acids cannot thus be separated, the purified nucleotide mixtures were then subjected to separation by liquid chromatography, under pressure, on an anion exchange resin in the Picker Nuclear nucleic acid analyzer, in order to determine the relative proportions of 2′-, 3′- and 5′-uridylic acids. The elution was performed with 0.001 *M* KH_2PO_4 buffer of pH 4.8.

Results and Discussion.—The first enzymic system chosen for the characterization of the behavior of the two enantiomeric ribonucleosides were the nucleoside phosphotransferases, a group of enzymes that have engaged the interest of this laboratory since their discovery.[1,2] The best investigated representative of this class of enzymes is the nucleoside phosphotransferase of carrot[1] which transfers organically bound phosphate to the 5′-position of a ribo- or deoxyribonucleoside. When L-uridine was compared with D-uridine as phosphate acceptor in this reaction, both substrates were found to be phosphorylated, but the affinity of the

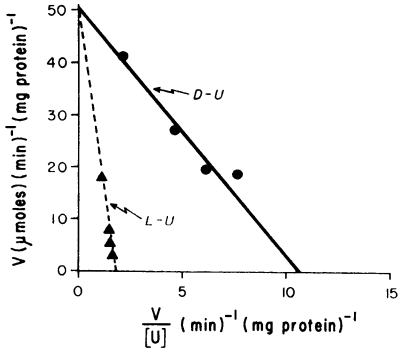


FIG. 1.—Determination of Michaelis constants for the nucleoside phosphotransferase of carrot with L-uridine and D-uridine as the acceptors. For L-uridine, $K_m = 28 \times 10^{-3}$ *M*; for D-uridine, $K_m = 5 \times 10^{-3}$ *M*.

enzyme for the D isomer was much greater than for the L isomer. As shown in Figure 1, D-uridine has a value of $K_m = 5 \times 10^{-3}$ M, L-uridine has $K_m = 28 \times 10^{-3} M$. The maximum velocity was the same for both enantiomers under the conditions of the experiment. At high acceptor concentrations both D- and L-uridine are phosphorylated with about equal efficiency.

The products of the enzyme action were isolated in both cases by paper chromatography and analyzed for homogeneity in the Picker Nuclear nucleic acid analyzer. Only 5'-uridine monophosphate was found.

It has long been known that the nucleoside phosphotransferase of human prostate is able to transfer phosphate not only to the 5' position but also to the 3' and 2' positions of a ribonucleoside.[13] An interesting difference emerged when the two enantiomeric nucleosides were subjected to the action of this enzyme. With D-uridine as the acceptor, all three possible nucleotides were formed, but with L-uridine only 5'- and 3'-L-uridylic acids were produced. A typical experiment is shown in Figure 2. More detailed information is provided in Table 2,

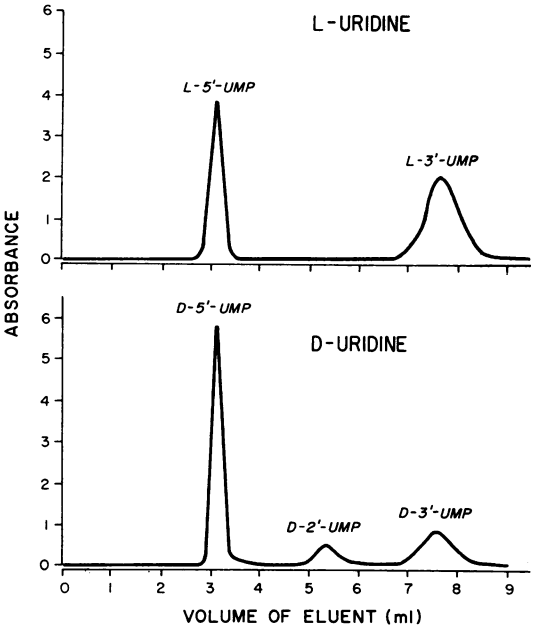


FIG. 2.—Nucleotides produced by the nucleoside phosphotransferase of human prostate through phosphate transfer to L-uridine and D-uridine. Liquid chromatography in the Picker Nuclear nucleic acid analyzer, with the elution profiles monitored at 254 mμ; the absorbance units are arbitrary. The identity of the individual elution peaks was verified with authentic nucleotide samples.

in which the proportions of the nucleotides formed from the two substrates at different periods of incubation are listed. Again, the affinity of the enzyme for the natural isomer is seen to be greater. The failure of L-uridine to accept phosphate in the 2' position is not without interest, since it points a way to exploring the active site of an enzyme through the use of suitable stereoisomeric substrates. It is not impossible that in our case the L-nucleoside, in contrast to the D-isomer, is held by the enzyme in such a manner as to render its 2' position inaccessible to the enzyme-donor complex.

TABLE 2. *Relative proportions of nucleotides produced through phosphate transfer to* L-*uridine or* D-*uridine by nucleoside phosphotransferase of human prostate.*

Acceptor	Incubation time (min)	Nucleotides Synthesized (% total nucleotides) 5'-UMP	3'-UMP	2'-UMP	Total nucleotides (% acceptor)
L-Uridine	10	34	66	0	2.1
	20	38	62	0	4.0
	30	42	58	0	4.4
	40	42	58	0	3.6
	50	54	46	0	2.8
	60	60	40	0	2.8
	120	89	11	0	2.2
D-Uridine	10	59	30	11	3.8
	20	62	27	11	5.0
	30	63	26	11	7.8
	40	63	26	11	7.1
	50	72	19	9	6.9
	60	72	17	11	6.6
	120	85	7	8	3.6

Concluding Remarks.—Surprisingly little work appears to have been done on the "unnatural" nucleosides and nucleotides. We are aware of the description of an L-deoxyriboside, L-thymidine,[14] of the riboside L-adenosine,[4, 15] and of L-adenylic acid.[15] Since, as this paper shows, the isomers of L-uridylic acid are accessible by enzymic phosphate transfer, several interesting lines of research can be envisioned: e.g., the synthesis of L-UDP and L-UTP with the aid of nucleotide kinases, the behavior of these substances as substrates of RNA polymerase and of polynucleotide phosphorylase, the study of L-polyuridylic acid and its biological activity, etc. We are engaged in these studies and hope to report on them later.

* This work has been supported by research grants from the National Institutes of Health and from the American Cancer Society. We are very grateful to Dr. Elinor F. Brunngraber for help and advice.

[1] Brunngraber, E. F., and E. Chargaff, *J. Biol. Chem.*, **242**, 4834 (1967).
[2] Boman, H. G., *Biochim. Biophys. Acta*, **16**, 249 (1955).
[3] Humoller, F. L., in *Methods in Carbohydrate Chemistry*, ed. R. L. Whistler and M. L. Wolfrom (New York and London: Academic Press, 1962), vol. 1, p. 83.
[4] Acton, E. M., K. J. Ryan, and L. Goodman, *J. Am. Chem. Soc.*, **86**, 5352 (1964).
[5] Recondo, E. F., and H. Rinderknecht, *Helv. Chim. Acta*, **42**, 1171 (1959).
[6] Yung, N., and J. J. Fox, in *Methods in Carbohydrate Chemistry*, ed. R. L. Whistler and M. L. Wolfrom (New York and London: Academic Press, 1963), vol. 2, p. 108.
[7] Nishimura, T., and I. Iwai, *Chem. Pharm. Bull. (Japan)*, **12**, 352 (1964).
[8] Nishimura, T., B. Shimizu, and I. Iwai, *Chem. Pharm. Bull. (Japan)*, **12**, 1471 (1964).
[9] Levene, P. A., *Nucleic Acids* (New York: Chemical Catalog Co., 1931), p. 170.
[10] Fox, J. J., and D. Shugar, *Biochim. Biophys. Acta*, **9**, 369 (1952).
[11] Volkin, E., and W. E. Cohn, in *Methods of Biochemical Analysis*, ed. D. Glick (New York and London: Interscience Publishers, 1954), vol. 1, p. 287.
[12] Brawerman, G., and E. Chargaff, *J. Am. Chem. Soc.*, **75**, 2020, 4113 (1953).
[13] Brawerman, G., and E. Chargaff, *Biochim. Biophys. Acta*, **15**, 549 (1954).
[14] Smejkal, J., and F. Šorm, *Coll. Czech. Chem. Commun.*, **29**, 2809 (1964).
[15] Asai, M., H. Hieda, and B. Shimizu, *Chem. Pharm. Bull. (Japan)*, **15**, 1863 (1967).